Plasmids Imparting Sulfonamide Resistance in *Escherichia coli*: Implications for Persistence[▽]

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Sulfonamide resistance remains prevalent among clinical isolates of *Escherichia coli* in the United Kingdom, despite a dramatic (>97%) national decline in the rate of prescription of sulfonamides in the 1990s. To investigate potential mechanisms accounting for this persistence, we characterized plasmids carrying *sul2*, the most prevalent determinant of sulfonamide resistance. Among 33 conjugative and 5 nonconjugative plasmids carrying *sul2*, resistance to other antimicrobial agents was common, but the spectrum of resistance profiles was diverse: 82%, 74%, and 45% carried resistance to ampicillin, streptomycin, and trimethoprim, respectively. Resistance to mercury was carried by 33% of the plasmids, but none conferred significant resistance to silver or to any of three disinfectants tested. The potential virulence genes *iutA* (aerobactin system) and *traT* (serum survival) were carried by 21% and 36% of the plasmids, respectively. The 33 conjugative plasmids belonged to five different incompatibility groups, FIB, B/O, I1, K/B, and P (42%, 33%, 9%, 3% and 3%, respectively), with 3 plasmids being unassigned, and to 19 similarity groups on the basis of their restriction profiles. The sequences flanking *sul2* were diverse and suggested more than one mechanism of genetic mobility. The five nonconjugative plasmids were all related to p9123 (pBP1), which was previously found to confer a fitness advantage on its host. We propose that the persistence of *sul2*, despite the reduced rate of prescription of sulfonamides, is due to a combination of coselection by antibiotics still in common use, a lack of a selective disadvantage in *sul2* carriage, and the genetic mobility of *sul2*.

Co-trimoxazole, a combination of sulfamethoxazole and trimethoprim, was introduced in the United Kingdom in 1969 and was widely used, becoming in particular a mainstay of treatment for urinary tract infections. In 1979, trimethoprim alone became available for the treatment of urinary tract infections, and in 1995, the licensed indications for co-trimoxazole were restricted in favor of the use of trimethoprim alone, primarily owing to concern about the rare side effects of sulfonamides (1). Despite the massive reduction in the rate of sulfonamide use that accompanied the switch in prescribing from co-trimoxazole to trimethoprim, resistance to sulfonamides has persisted at high rates among clinical isolates of *Escherichia coli* (2, 6).

Three acquired genes imparting sulfonamide resistance have been described in *E. coli*; of these, only two (*sul1* and *sul2*) are prevalent in human isolates, including those from east London, United Kingdom (2, 6). The *sul2* gene has remained the more prevalent over time and is found both alone and in combination with *sul1*. While *sul1* is typically associated with class 1 integrons (26), *sul2* is generally not considered part of a distinct genetic element, although it is frequently found adjacent to the streptomycin resistance gene pair *strAB* (8, 23, 25). We have previously demonstrated that clinical isolates of *E. coli* resistant to sulfonamides frequently carried genes for resistance to other antimicrobial agents but had not established whether the genes for resistance to multiple compounds were

carried on plasmids that were the same as or separate from those that carried the *sul* determinants (2, 6). We also determined that a small nonconjugative plasmid carrying *sul2*, p9123, conferred a fitness advantage on its host, whereas three other plasmids carrying *sul2* had a fitness cost (5). In the study described here, we have further characterized plasmids carrying *sul2* among clinical isolates of *E. coli*. We investigated the genetic linkage of *sul2* with resistance to other antimicrobials and xenobiotics and assessed the genetic diversity of these plasmids as a marker for the mobility of the *sul2* gene.

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MATERIALS AND METHODS

Bacterial isolates and antimicrobial susceptibility testing. This study used a collection of *E. coli* isolates collected at the Royal London Hospital in 1999 and described previously (6). Antibiotic susceptibility was determined by the disc diffusion assay, in accordance with the guidelines of the British Society for Antimicrobial Chemotherapy (3). Antibiotic discs were obtained from Oxoid, Basingstoke, United Kingdom. The MICs of heavy metals (mercury chloride and silver nitrate; Sigma, Poole, United Kingdom) and detergents (triclosan, chlorhexidine, and cetrimide; Sigma) were determined by broth microdilution, according to the guidelines of EUCAST for antimicrobial susceptibility testing (7).

Plasmid characterization and manipulation. Plasmid DNA was extracted by the method of Kado and Liu (12) (for visualization only) or the alkaline lysis method (24). SacI-digested plasmid DNA was separated with a CHEF DRII apparatus (Bio-Rad, Hemel Hempstead, United Kingdom) at 6 V/cm and with ramping from 0.1 s to 1.0 s over 8 h. Conjugation was by the plate-mating protocol of Livermore and Williams (16) with *E. coli* J62-1 (nalidixic acid resistant, β-galactosidase negative) as the recipient. Nonconjugative plasmids were introduced into *E. coli* J62-1 by electrotransformation (24). The transconjugants and transformants were selected on Iso-Sensitest agar (Oxoid) with sulfamethoxazole (500 μg/ml; Sigma) and nalidixic acid (100 μg/ml; Sigma). For the cloning

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of *sul2*, plasmid DNA was digested with BamHI, ligated into pGEM-3Z (Promega, Southampton, United Kingdom), transformed into *E. coli* JM109 competent cells (Promega), and selected on Iso-Sensitest agar (Oxoid) with sulfamethoxazole (500 μg/ml; Sigma) and ampicillin (100 μg/ml; Sigma). Southern transfer was performed by standard techniques (24). Probes were labeled and detected with the PCR DIG probe synthesis kit and the DIG DNA labeling and detection kit (Roche Diagnostics, Lewes, United Kingdom), respectively.

PCR amplification and sequencing. PCR was performed with boiled cell lysates by using the ReddyMix master mixture (Abgene, Epsom, United Kingdom). Sequencing was conducted at the Genome Centre of Barts and The London Hospital. Plasmids were assigned to incompatibility groups by using the multiplex PCR described by Johnson and colleagues (11), and the assignments were confirmed by using single primer pairs (4). Virulence genes were detected as described by Johnson and Stell (10). The other primers used for PCR and sequencing are listed here: sulfonamide resistance gene primers sul1-F (5′-CCGATATTGCTGAGGCGG-3′; GenBank accession no. U42226; nucleotides 435 to 453), sul1-R (5′-CCAACGCCGACTTCAGCT-3′; GenBank accession no. U42226; nucleotides 684 to 702), sul2-F (5′-TCGTCAACATAACCTCGGACAG-3′ [6]), sul2-R (5′-GTTGCGTTTGATACCGGCAC-3′ [6]), sul3-F (5′-GAGCAAGATTTTTGGAATCG-3′ [19]), and sul3-R (5′-CTAACCTAGGGCTTTGGA-3′ [19]); streptomycin resistance gene primers strA-F (5′-CAACTGGCAGGAGGAACA-3′ [15]), strA-R (5′-CGCAGATAGAAGGCAAGG-3′ [15]), strB-F (5′-TTCTCATTGCGGACACCT-3′ [15]), strB-R (5′-GGCATTGCTCATCATTTG-3′ [15]), aadA1-F (5′-TATTGATTTGCTGGTTACGG-3′ [15]), and aadA1-R (5′-CGCTATGTTCTCTTGCTTTT-3′ [15]); upstream sul2 primers sul2 out (5′-CCTGCGCAATGGCTGCGTCTGG-3′; GenBank accession no. AY360321; nucleotides 102 to 124), sul2 up Type 1 (5′-TCTTCCGAACATGACACCAGTC-3′; GenBank accession no. AY360321; nucleotides 5980 to 6002), sul2 up Type 2 (5′-ACAGCTCCATAGGCCGCTTTCC-3′; GenBank accession no. M28829; nucleotides 7712 to 7734), sul2 up Type 2A (5′-GCCATTTCAGCGGCTGTGATGC-3′; GenBank accession no. AB109805; nucleotides 3555 to 3577); sul2 up Type 2B (5′-AGTGGTTCGACCATGCGCAAGC-3′; GenBank accession no. M28829; nucleotides 7435 to 7457), sul2 up Type 2C (5′-AAGTAGTAACCCACCTCCGCAG-3′; GenBank accession no. AY055428; nucleotides 21478 to 21500), sul2 up Type 2D (5′-CTGATGGTGCCCGAAAACTATA-3′; GenBank accession no. NC_007365; nucleotides 59902 to 59924), sul2 up Type 3 (5′-TGGTAAATATCGCCATGGGCAG-3′; GenBank accession no. AJ319822; nucleotides 1532 to 1554), repA-F (5′-GCTGACGGCCTGCTGATCCAGC-3′; GenBank accession no. M28829; nucleotides 6195 to 6217), repC-F (5′-ACGAACCCGGCGCACTGTCTG-3′; GenBank accession no. M28829; nucleotides 6758 to 6778), repC-R (5′-TGCTGATGCGCACATGCTGG-3′; GenBank accession no. M28829; nucleotides 7281 to 7301), and trbC-out (5′-AGGTGGAAAACAAGAGAGTCG-3′; GenBank accession no. NC_007365; nucleotides 59623 to 59644); and miscellaneous primers intI1-F (5′-GCGTGATTGTATCTCACT-3′ [2]), intI1-R (5′-GACGCTCCTGTTGCTTCT-3′ [2]), blaTEM-F (5′-ATGATGATTCAACATTTCCG-3′ [15]), and blaTEM-R (5′-CCAATGCTTAATCAGTGACG-3′ [15]).

RESULTS AND DISCUSSION

Genetic linkage to other resistance determinants. Sulfonamide resistance plasmids bearing *sul2* were transferred into a susceptible (plasmid-free) host, *E. coli* J62-1, in order to explore the cocarriage of genes for resistance to other compounds in a standard genetic background. One hundred thirteen independent *sul2*-containing *E. coli* isolates were investigated, of which 32 (28%) also carried *sul1* (6). Conjugative plasmids were successfully transferred from 41 (36%) isolates, but in 8 of these the plasmid carrying *sul2* was consistently cotransferred with another plasmid so they were not investigated further. Among the 33 conjugative plasmids that transferred independently of other plasmids, 8 (24%) carried both *sul1* and *sul2* and 25 (76%) carried *sul2* only; none carried *sul3*. The transfer of plasmids by transformation was attempted with DNA extracted from the isolates that failed to conjugate and was successful with the DNA from five isolates; all five transformed (nonconjugative) plasmids carried *sul2* but not *sul1* or *sul3*.

All 33 conjugative and 5 nonconjugative plasmids conferred at least one additional resistance trait besides sulfonamide resistance to the *E. coli* recipient, but the spectrum of resistance patterns was diverse (Table 1). The most frequently occurring coresistances were to ampicillin (82%), streptomycin (74%), and trimethoprim (45%). These results suggest that the continued extensive use of ampicillin (and related β-lactam antibiotics) and trimethoprim might coselect for sulfonamide resistance.

The plasmids were tested by PCR for the presence of bla_{TEM}, a likely determinant of ampicillin resistance, and *strAB* and *aadA1*, the two principal determinants of streptomycin resistance in *E. coli*. There was a complete correlation between the detection of bla_{TEM} and ampicillin resistance. The *strA* and *strB* genes were detected in 21 (55%) plasmids, all of which conferred streptomycin resistance. *strA* alone was detected in a further six plasmids; streptomycin resistance was still observed in the corresponding transconjugants, albeit with increased zone diameters in some cases (e.g., p99163 and p99164). *strB* alone was detected in two plasmids, neither of which imparted streptomycin resistance. The *aadA1* gene was identified in five plasmids, yet three of these did not impart streptomycin resistance, as determined by disc diffusion. The plasmids were also tested for the presence of *intI1*; this gene encodes the integrase of type 1 integrons and is frequently associated with *sul1*, which forms part of the 3′ conserved region of these elements (26). It was detected in 14 plasmids, including all 8 *sul1*-containing plasmids. Six plasmids contained *intI1* without *sul1*, suggesting a rearrangement of the typical class 1 integron, possibly comparable to previously described variants in which *sul1* has been lost (18).

The transconjugants and the transformants were tested for decreased susceptibility, relative to the susceptibility for the plasmid-free recipient, to various xenobiotics. Twelve plasmids (32%) conferred increased resistance to mercuric chloride (MICs, 32 μg/ml and 4 μg/ml, respectively). None of the plasmids conferred resistance to silver nitrate (MICs, 4 μg/ml both with and without plasmids). Similarly, no appreciable resistance was detected to chlorhexidine, triclosan, or cetrimide, which represent the bisguanide, bisphenol, and quaternary ammonium classes of disinfectants, respectively (median MICs, 0.5, 0.5, and 12 μg/ml, respectively; the MICs did not differ by more than 1 twofold dilution in any transconjugant or transformant). Thus, among all these agents, only mercury might potentially have a role in coselection for sulfonamide resistance. An association between mercury resistance and multiple-antimicrobial resistance in clinical *E. coli* isolates has been demonstrated previously, although resistance to sulfonamide was not recorded (21). The installation of amalgam fillings in primates has been reported to result in the release of significant quantities of mercury and might select for increased mercury resistance among intestinal flora, although this is controversial and a study with children did not find any difference in the prevalence of mercury-resistant flora among those with and without amalgam fillings (20, 27).

Genetic linkage to other selective markers. In addition to linkage to resistance, we considered the possibility that *sul2* could be genetically linked to other determinants that might give isolates a selective advantage in the host. We therefore investigated whether a range of putative or established viru-

TABLE 1. Properties of *sul2*-containing plasmids

Plasmid type and name	Restriction profile	Incompatibility group	Plasmid size (kb)	*sul2* fragment size (kb)[a]	Flanking sequence Upstream	Downstream	A	S	W	T	C	K	Hg²⁺	TEM	*strA*/*strB*	*aad*	*cvaC*	*iutA*	*traT*	Presence of *intI1*
Plasmids with *sul2* only																				
p99015	Z1	FIB	118	36	2B, *repC*[c]	*strA*	R	R	–	R	R	–	R	+	+/+	–	–	–	–	–
p99018	Z1	FIB	118	36	2B, *repC*	*strA*	R	R	–	R	R	–	R	+	+/+	–	–	–	–	–
p99019	Z2	FIB	126	ND[d]	2B, *repC*	*strA*	R	R	–	R	R	–	R	+	+/+	–	–	–	+	–
p99026	Y	ND	73	ND	2A	*strA*	R	R	–	–	–	–	–	+	+/+	–	–	–	+	–
p99034	X1	FIB	119	ND	2B, *repC*	*strA*	R	R	R	–	–	–	R	+	+/+	–	–	+	+	+
p99272	X2	FIB	119	33	2B, *repC*	*strA*	R	R	R	R	–	–	R	+	+/+	–	+	+	+	+
p99290	X2	FIB	119	33	2B, *repC*	*strA*	R	R	R	–	–	–	R	+	+/+	–	–	+	+	+
p99339	X3	FIB	137	33	2B, *repC*	*strA*	R	R	R	–	–	–	R	+	+/+	–	–	+	+	+
p99135	W1	I1	76	22.5	2B	ND	R	–	–	–	–	–	–	+	–/–	–	–	–	–	–
p99136	W2	I1	100	22.5	2B	ND	R	–	–	–	–	–	–	+	–/–	–	–	–	–	–
p99332	V	FIB	107	35	2B	*strA*	R	R	–	R	R	–	R	+	+/+	–	+	+	+	–
p99288	U	FIB	101	ND	2B	ND	R	R	–	–	–	–	–	+	–/–	+	+	+	+	+
p99054	T1	ND	45	35	2B	ND	R	–	–	–	–	–	–	+	–/–	–	–	–	–	–
p99082	T1	ND	45	35	2B	ND	R	–	–	–	–	–	–	+	–/–	–	–	–	–	–
p99009	S1	B/O	97	5.1	2D, *trbC*	*glmM*	–	–	R	R	–	–	–	–	–/–	+	–	–	–	–
p99051	S2	B/O	75	4.5	2D, *trbC*	*strA*	R	R	–	–	–	–	–	+	+/–	–	–	–	–	–
p99057	S3	B/O	83	4.5	2D, *trbC*	*strA*	R	R	–	–	–	–	–	+	+/–	–	–	–	–	–
p99163	S4	B/O	80	4.5	2D, *trbC*	*strA*	R	R	–	–	–	–	–	+	+/–	–	–	–	–	–
p99164	S5	B/O	77	4.5	2D, *trbC*	*strA*	R	R	–	–	R	–	–	+	+/–	–	–	–	–	–
p99372	S6	B/O	81	4.5	2D, *trbC*	*strA*	R	R	–	–	–	–	–	+	+/–	–	–	–	–	–
p99171	R	B/O	97	4.5	2D, *trbC*	*strA*	R	R	R	–	–	–	–	+	+/–	–	–	–	–	+
p99130	Q	FIB	72	6	2A	*strA*	R	R	R	R	R	–	–	+	+/+	–	–	+	+	–
p99151	P	FIB	136	4.9	2B, *repC*	*strA*	R	R	–	R	R	–	R	+	+/+	–	–	–	+	–
p99197	O	B/O	110	25	2B, *repC*	ND	R	–	R	–	–	R	R	+	–/+	–	–	–	–	–
p99246	N	K/B	109	8	2D, *trbC*	ND	–	–	R	–	–	–	–	–	–/–	+	–	–	–	–
Plasmids with *sul1* and *sul2*																				
p99030	A1	B/O	104	3.8	2D, *trbC*	*strA*	R	R	R	–	–	–	–	+	+/+	–	–	–	–	+
p99309	A1	B/O	104	3.8	2D, *trbC*	*strA*	R	R	R	R	–	–	–	+	+/+	–	–	–	–	+
p99145	A2	B/O	94	3.8	2D, *trbC*	*strA*	–	R	R	–	–	–	–	–	+/+	–	–	–	–	+
p99170	B	I1	102	13.5	2B	ND	–	–	R	–	–	–	–	–	–/–	+	–	–	–	+
p99184	C	FIB	156	17.5	2B, *repC*	*strA*	R	R	–	R	–	–	–	+	+/+	–	–	+	+	+
p99323	D	FIB	144	6.5	2B, *repC*	*strA*	R	R	R	–	R	–	–	+	+/+	–	–	–	+	+
p99376	E	P	112	17.5	2B, *repC*	*strA*	R	R	R	R	–	–	R	+	+/+	+	–	–	–	+
p99378	F	FIB	102	ND	2B, *repC*	*strA*	R	R	R	–	–	–	R	+	+/+	–	+	–	+	+
Nonconjugative plasmids with *sul2*																				
p99249	G1		6.2		*orf5*	*strA*	–	R	–	–	–	–	–	–	+/+	–	–	–	–	–
p99257	G1		6.2		*orf5*	*strA*	–	R	–	–	–	–	–	–	+/+	–	–	–	–	–
p99314	G1		6.2		*orf5*	*strA*	–	R	–	–	–	–	–	–	+/+	–	–	–	–	–
p99187	G2		7.0		*orf5*	*strA*	R	–	–	–	–	–	–	+	+/+	–	–	–	–	–
p99253	G3		9.0		*orf5*	ND	R	–	R	–	–	–	–	+	–/+	–	–	–	–	–

[a] The size of the SacI restriction fragment to which the *sul2*-specific probe hybridized.
[b] A, ampicillin; S, streptomycin; W, trimethoprim; T, tetracycline; C, chloramphenicol; K, kanamycin; R, resistant; –, no resistance.
[c] Amplified with primer repC-F, in addition to primer 2B.
[d] ND, not determined, but amplification with the *strA*-specific primer was not successful.

lence genes that have previously been associated with plasmid carriage (10) could be detected on the 33 conjugative plasmids. The genes *afa*/*draBC* and *bmaE* were not detected on any of the plasmids. The genes *cvaC* (colicin V, used as a marker for ColV virulence plasmids [10]), *iutA* (aerobactin siderophore system), and *traT* (serum survival) were detected on 4, 7, and 13 (11%, 18% and 34%) of the plasmids, respectively. The prevalence of these five potential virulence genes among conjugative plasmids carrying *sul2* was comparable to or lower than their overall prevalence reported previously among 74 urine isolates of *E. coli* (9), suggesting no coselection of *sul2* with virulence factors.

Genetic background of *sul2*. A further factor likely to affect the long-term persistence of *sul2* is the frequency with which it transfers to new genetic backgrounds and, in particular, to new plasmids. *sul2* has most commonly been found as part of a cassette structure adjacent to the streptomycin resistance genes *strA* and *strB*. This cassette is not recognized as a mobile element in its own right but has been associated with transposition mediated by other elements, such as SXT, IS*26*, and ISCR2 (17, 29, 30). As a marker for the extent of the long-term mobility of *sul2*, we investigated the diversity of plasmid carrying *sul2* by PCR-based determination of their incompatibility groups and comparison of their restriction profiles.

Replicon typing (4, 11) of the 33 conjugative plasmids demonstrated five different incompatibility groups (Table 1). Groups FIB (14 plasmids) and B/O (11 plasmids) were the most common, while groups I1, P, and K/B were represented only rarely (1 to 3 plasmids each). No replicon product was obtained for three plasmids.

The SacI restriction profiles (Table 1) of the 33 conjugative plasmids gave 29 distinct profiles that differed by at least one band. The use of pulsed-field gel electrophoresis allowed the excellent separation of bands in the range of 2 to 50 kb (data
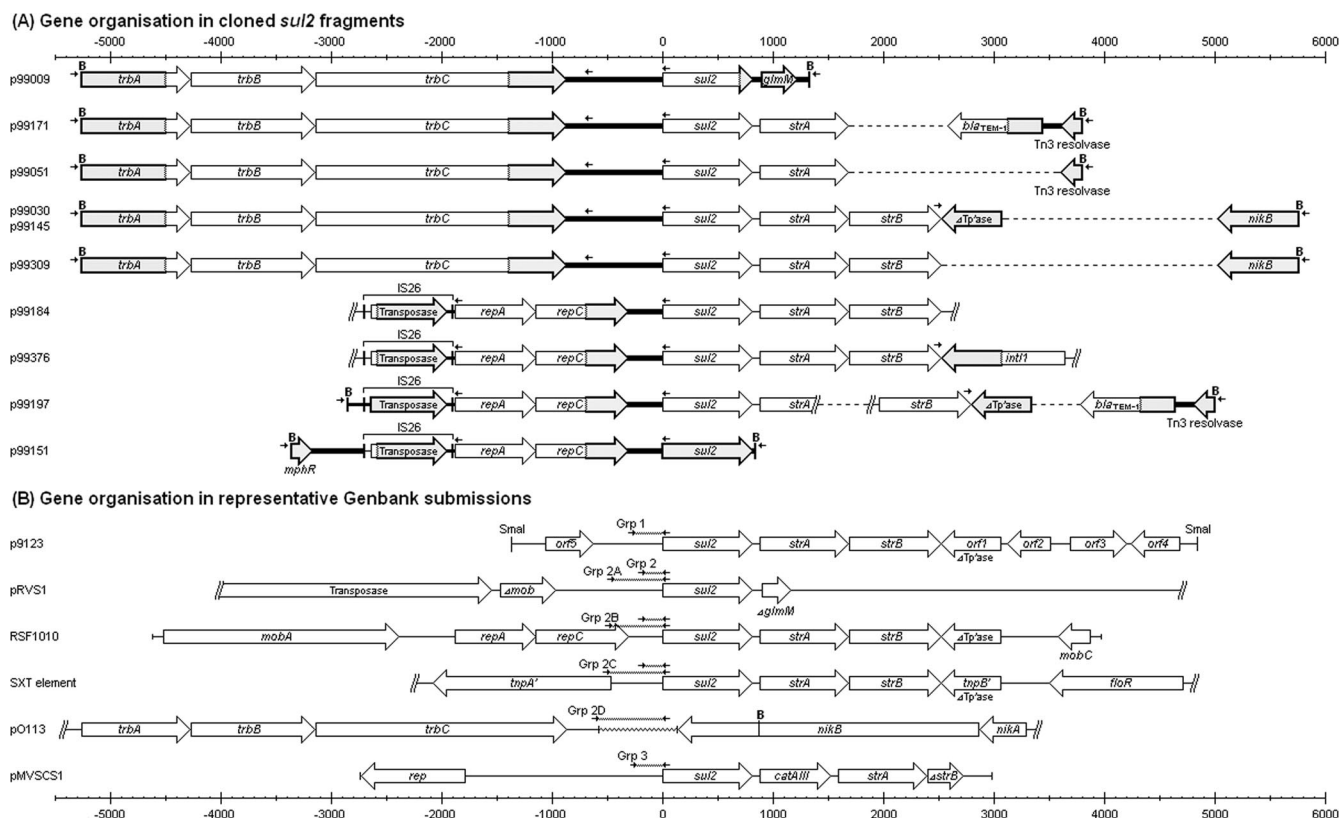
FIG. 1. Organization of genes adjacent to *sul2*. (A) BamHI fragments cloned from conjugative *sul2*-containing plasmids. The sequenced regions are indicated by bold lines and are filled in gray, with the positions of the sequencing primers indicated by small arrows. Homology to other genes was determined by the amplification of overlapping PCR products of the predicted length (*repA*, *repC*, *sul2*, *strA*, *strB*) and by partial restriction site conservation (*trbA-trbC*). Dotted lines represent uncharacterized DNA. The scale is in bp. B, BamHI restriction sites. (B) Representative GenBank submissions with different *sul2* genes in different contexts. The positions of the diagnostic PCR products used to distinguish the upstream regions are indicated above the sequences. For pO113, the serrated line indicates the region that is replaced by a sequence that includes *sul2* in plasmids of group (Grp) 2D.

not shown). These profiles formed 19 similarity groups (each profile within a group differed by no more than three bands from another one in the group). Plasmids belonging to the same restriction group also belonged to the same incompatibility group (as determined by PCR) and generally had the same or similar resistance profiles and virulence gene profiles. In contrast, there was wide diversity in the resistance and the virulence gene profiles between restriction groups, whether or not these shared the incompatibility group. Similarly, the size of the SacI restriction fragment that hybridized to the *sul2* probe correlated closely with the restriction group but indicated diversity between groups in the sequences surrounding *sul2*.

The five nonconjugative plasmids belonged to a single similarity group; three were indistinguishable from previously described plasmid p9123, whose restriction profile corresponds to that of pBP1 (5), while the other two carried additional resistance genes that coincided with their larger sizes.

Analysis of regions flanking *sul2*. The finding of five different incompatibility groups among conjugative plasmids carrying *sul2*, in addition to pBP1-like plasmids, indicates that *sul2* has been able to transfer into multiple plasmid backbones. The restriction profile diversity of plasmids carrying *sul2* even within the two most prevalent incompatibility groups further

suggests either that *sul2* has been mobilized into these groups on multiple occasions or that the plasmid backbones have diversified extensively since the acquisition of *sul2*. To investigate the variation in the genetic environment of *sul2*, the upstream sequences of 25 *sul2* genes deposited in the GenBank database were aligned by using ClustalW software (28). Within 400 bp upstream of the *sul2* start codon, 12 different sequences were found, and these diverged at multiple positions. On the basis of the most frequently represented sequences, PCR primers were developed to characterize regions flanking *sul2* in our plasmids (Fig. 1). Upstream sequences were classified as types 1, 2 (2A, 2B, or 2C), and 3, with the subtypes of type 2 having a conserved sequence of 302 bp immediately upstream of *sul2* and subsequently diverging.

All 5 of the nonconjugative plasmids (restriction profile G) and none of the 33 conjugative plasmids contained the type 1 sequences found upstream of *sul2* in small plasmids typified by pBP1 (5). None of the plasmids contained the type 3 upstream sequences observed in pMVSCS1 and other small plasmids that have been identified in the members of the family *Pasteurellaceae* (13). However, in all conjugative plasmids, a primer pair designed to amplify the type 2 sequence 162 bp upstream of *sul2* in small plasmids typified by RSF1010 (22) gave an amplification product of the expected size. Of these, 20 were

further classified as type 2B, having homology to RSF1010 for at least 439 bp upstream of *sul2*, and two were classified as type 2A, having an alternative upstream sequence previously described in several plasmids from *Vibrio* spp. and the *Pasteurellaceae*. None of the plasmids had the type 2C sequence, as described in the *Vibrio cholerae* SXT resistance element (30), while in 11 plasmids of four restriction groups, the sequence upstream was not amplified by type 2A, 2B, or 2C primers.

In order to look more closely at the DNA flanking *sul2*, BamHI restriction fragments containing the gene were cloned from a subset of conjugative plasmids and the relevant sequences were determined (Fig. 1). In four plasmids having an upstream type 2B sequence but different restriction profiles and belonging to three incompatibility groups (groups FIB, B/O, and P), sequencing of about 700 bp upstream of *sul2* confirmed approximately 99% identity to the corresponding sequence of RSF1010. Further analysis by amplification of the overlapping PCR products confirmed that homology to RSF1010 extended to the 5′ end of *repA*, while sequencing out from this gene identified an adjacent copy of the IS*26* transposase in all cases (Fig. 1). In three of these plasmids, *strA* and *strB* were identified downstream of *sul2* by overlapping PCR, which matches the organization of RSF1010, but the plasmids diverged from each other and from RSF1010 at the 3′end of *strB*. These three plasmids of different incompatibility groups therefore appear to represent independent insertions of a large segment of RSF1010 adjacent to (or together with) a copy of IS*26*. (In the fourth plasmid of this group, a BamHI site had arisen downstream of *sul2*, and the relative locations of *strA* and *strB* were not determined.) However, not all conjugative plasmids with a type 2B upstream sequence had the same extent of homology to RSF1010, as only 13 of 20 yielded a PCR amplification product with primers spanning from the middle of *repC* to *sul2* (Fig. 1 and Table 1).

Subcloned BamHI fragments from six plasmids with unknown type 2 upstream sequences were characterized; these belonged to three different groups by restriction profile analysis, but all were incompatibility group B/O. All six plasmids revealed the same genetic arrangement upstream of *sul2* (Fig. 1). Immediately upstream of *sul2*, a sequence of 302 bp was identical to the sequence of RSF1010. (An apparent recombination site at 302 bp upstream of *sul2* was also observed in several other sequences in the GenBank database compared to the sequence of RSF1010.) This was followed by a 274-bp sequence with no significant homology to any sequence in the GenBank database. Then, from 583 bp upstream of *sul2* lay a sequence with 97% identity to *trbC* in the transfer region of *E. coli* plasmid pO113, which in turn shares homology with IncI plasmid R64; neither pO113 nor R64 carries *sul2* at this position (14). PCR amplification with primers spanning from the 3′ end of *trbC* to the 5′ end of *sul2* demonstrated that the same upstream sequence (designated type 2D) was present in all 11 plasmids with an unknown type 2 region (Table 1). Downstream of *sul2* there was considerable diversity among the group B/O (type 2D) plasmids, including disruption of the conserved *strA-strB* gene arrangement in some cases (Table 1 and Fig. 1). We speculate that diversification subsequent to the acquisition of *sul2* may explain the restriction fragment polymorphism among many of the incompatibility group B/O plasmids.

Upstream sequences among plasmids not transferred. Since we had been unable to transfer plasmids from a number of clinical isolates, we considered whether they might contain *sul2* in similar or different genetic contexts. We therefore examined flanking sequences upstream of *sul2* in 35 clinical isolates from which plasmids had not been transferred. Among these isolates, 3 (8%) had type 1, pBP1-like sequences; 30 (86%) had type 2 sequences, as detected in all conjugative plasmids; and in 2 (6%), neither of these upstream sequences could be detected. Of the isolates with type 2 sequences, 17 isolates (57% of 30 isolates) had the type 2B upstream sequence indicating homology to RSF1010, 6 isolates (20%) had the type 2D upstream sequence, 1 isolate (3%) had the type 2A upstream sequence, and 6 isolates (20%) gave positive results with the type 2 primer but did not amplify with the type 2A, 2B, and 2D primers (the type 2C primer was not tested with these isolates). We conclude that the upstream sequences observed among the 38 nonconjugative and conjugative plasmids that we have characterized in detail are broadly representative of those in the *E. coli* population investigated but that there is likely to be additional diversity in the population among plasmids that we have not been successful in transferring.

Factors involved in the persistence of *sul2*. It is likely that the carriage of *sul2* on plasmids that also determine resistance to antimicrobials still in common use (such as ampicillin) contributes to the maintenance of sulfonamide resistance in the United Kingdom *E. coli* population via coselection. However, the findings of this study do not support the hypothesis that sulfonamide resistance is maintained through coselection by other xenobiotics, such as disinfectants or heavy metals, nor is it maintained through linkage with known virulence factors.

The diversity of restriction profiles and *sul2* flanking sequences among the plasmids characterized in the present study demonstrates that *sul2* has a history of genetic mobility. In some cases, mobility appears to be associated with IS*26*, while the presence of a common recombination site at 302 bp upstream of *sul2* may indicate another mechanism of genetic transfer that is currently unclear. The persistence of *sul2* over time represents a balance between the rate of loss of the gene by selective disadvantage, deletion from plasmids, and the loss of plasmids carrying *sul2* versus the rate of gain by selective advantage (including fitness advantage, as observed with p9123 [5]), transfer to new plasmids, and the transfer of plasmids carrying *sul2*. Although we can put no time scale on the rate of *sul2* transfer to new plasmids, we suggest that its evident mobility will contribute to its persistence in the bacterial population. We would predict that other resistance genes that have become very widely established within their host populations, particularly where resistance is carried on diverse mobile elements, will be similarly persistent unless their carriage represents a significant selective disadvantage.

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